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Characterization of the 5′ flanking region of the human D_{1A} dopamine receptor gene

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ABSTRACT **To study how the expression of the D_{1A} dopamine receptor gene is regulated, a human genomic clone was isolated by using a rat cDNA as probe. A 2.3-kilobase genomic fragment spanning −2571 through −236 relative to the adenosine of the first methionine codon was sequenced. The gene has an intron of 116 base pairs in the 5′ noncoding region, nucleotides −599 through −484 as determined by S1 mapping and reverse transcription–PCR. It has multiple transcription initiation sites located between −1061 and −1040. The promoter region lacks a TATA box and a CAAT box, is rich in G+C content, and has multiple putative binding sites for transcription factor Sp1. Thus, the promoter region of the human D_{1A} gene has features of "housekeeping" genes. However, it also has consensus sequences for AP1 and AP2 binding sites and a putative cAMP response element. The ability of four deletion mutants of the 2.3-kilobase fragment to modulate transcription of the heterologous chloramphenicol acetyltransferase gene in the promoterless plasmid pCAT-Basic was determined. All mutants demonstrated substantial transcriptional activity in the murine neuroblastoma cell line NS20Y, which expresses the D_{1A} gene endogenously. Transient expression assays suggested the presence of a positive modulator between nucleotides −1340 and −1102, and a negative modulator between −1730 and −1341. The four genomic fragments had no or very low transcriptional activity in NB41A3, C6, and Hep G2 cells, which are not known to express this gene. Thus, the human D_{1A} gene belongs to the category of tissue-specific, regulated genes that have housekeeping-type promoters.**

Dopaminergic transmission plays a central role in the generation of coordinated motor function, neuroendocrine modulation, and perhaps behavior and cognition (1). The biochemical and cellular effects of dopamine are mediated through its cell surface receptors, which belong to a large superfamily of receptors coupled to guanine nucleotide-binding proteins (2). Disturbances in this neurotransmitter and its receptors have been implicated in a number of neurobehavioral disorders, including Parkinson disease and schizophrenia as well as complications of their long-term therapy (3–7). Yet the mechanisms underlying these alterations remain to be elucidated. Analysis of the molecular regulation of dopamine receptors should help advance our understanding of how they are modified by disease and by pharmacologic manipulation.

At least five different dopamine receptor genes have been identified. Their coding regions have been sequenced, but their 5′ upstream portions and transcriptional regulation have not been addressed. Among the different dopamine receptors the D_{1A} and the D_2 receptors are the primary ones expressed in the striatum. Although the motor and behavioral effects of dopaminergic transmission have been traditionally attributed to actions at the D_2 receptor, the functional significance of the D_1 subclass has gained increasing attention in recent years. The adenylate cyclase-linked D_{1A} receptor has been cloned and the coding region found to be intronless (8–11). The human gene has been mapped on chromosome 5 (10). To begin to unravel the molecular events involved in the transcriptional regulation of this gene, we have characterized a genomic clone encoding the human D_{1A} dopamine receptor. The sequence of a 2.3-kilobase (kb) stretch of the 5′ flanking region was analyzed, the transcription initiation sites were determined, and the ability of various deletion mutants to direct transcription of a heterologous gene was studied.

MATERIALS AND METHODS

Screening of Genomic Library. A human placental genomic library constructed in the λ phage vector EMBL3/SP6/T7 (Clontech) was screened with the rat D_{1A} dopamine receptor cDNA pB73D1 (11) under high-stringency hybridization conditions. Among the 11 positive clones identified, 1 had a 5.3-kb *Sac* I fragment that gave the strongest hybridization signal on Southern blot analysis using a nick-translated 1.3-kb *Eco*RI fragment of pB73D1 as the probe. This *Sac* I fragment was subcloned into pGEM-3Zf(−) (Promega), giving plasmid pD1-5.3, for further characterization.

DNA Sequencing. The 3.0-kb *Pst* I–*Sac* I fragment of pD1-5.3, which was shown to represent the transcribed region of the gene by restriction analysis and Southern blots, was excised and the plasmid was religated, leaving a 2.3-kb *Sac* I–*Pst* I insert in pGEM-3Zf(−), giving pD1G3. Both strands of this 2.3-kb fragment were sequenced. Oligonucleotide primers were synthesized according to vector-specific sequences and prior sequence information. Sequencing was done by the Sanger dideoxynucleotide chain-termination method with Sequenase (United States Biochemical) using 7-deaza-dGTP (12).

Reverse Transcription and PCR. Total RNA was isolated from postmortem human caudate tissue using the guanidinium thiocyanate/CsCl method (13). Poly(A)$^+$ RNA was purified by using a Poly(A) Quik mRNA isolation kit (Stratagene). Two micrograms of poly(A)$^+$ RNA was reverse-transcribed with avian myeloblastosis virus reverse transcriptase (cDNA Cycle kit, Invitrogen) after overnight hybridization with 10 pmol of PS4 primer (Fig. 1*B*). After removal of excess primer with Centricon 100 (Amicon), the volume was adjusted to 300 μl and 10 μl was used as template in PCR. Primer D1-3′, which is complementary to the nucleotide sequence from −408 to −426, was used as the 3′ primer in each PCR. Oligonucleotides used as 5′ primers are indicated in the legend to Fig. 2.

S1 Nuclease Mapping Probes. Single-stranded genomic DNA was prepared from pD1G3 and used as template in

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Abbreviations: CAT, chloramphenicol acetyltransferase; RACE, rapid amplification of cDNA ends.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M85247).

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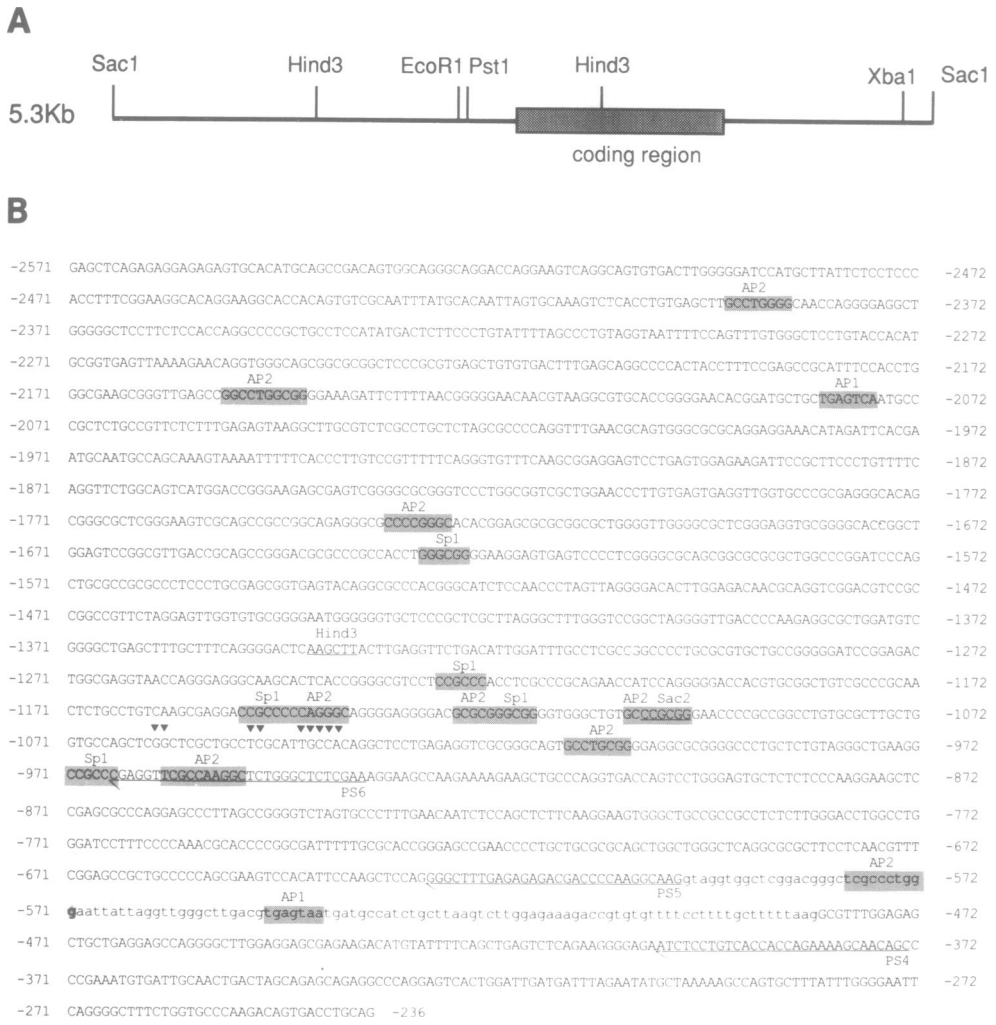

FIG. 1. Restriction map and nucleotide sequence of the human D_{1A} genomic clone. (*A*) Restriction map of the 5.3-kb *Sac* I fragment that hybridized with the rat D_{1A} cDNA probe. The 3.0-kb *Pst* I–*Sac* I fragment was excised from the plasmid and the remaining 2.3-kb *Sac* I–*Pst* I fragment was sequenced. (*B*) Nucleotide sequence of the 5′ untranslated region. Numbers are relative to the adenosine in the first ATG codon (8–10). Three primers (PS4, PS5, and PS6) used in reverse transcription, S1 mapping, and 5′-RACE are shown by horizontal arrows below the sequence. The *Hin*dIII and *Sac* II sites are underlined. Putative sequences for AP1, AP2, and Sp1 transcription factor binding sites are shaded. Arrowheads indicate transcription start sites determined by S1 mapping. Lowercase characters from −599 to −484 denote the intron I sequence.

primer extension reactions with ^{32}P-end-labeled synthetic 30-mer oligonucleotide primers PS4, PS5, and PS6 (Fig. 1*B*). PS4 is complementary to bases −373 to −402 (5′-GCTGTTGCTTTTCTGGTGGTGACAGGAGAT-3′), PS5 is complementary to bases −600 to −629 (5′-CTTGCCT-TGGGGTCGTCTCTCTCAAAGCCC-3′), and PS6 is complementary to bases −937 to −966 (5′-TTCGAGAGCCCA-GAGCCTTGGCGAACCTCG-3′). Primer (5 pmol) and single-stranded DNA (5 pmol) were denatured at 65°C for 5 min, annealed at 45°C for 15 min, and extended in 55 μl of *Taq* polymerase buffer with 2.5 units of *Taq* polymerase at 40°C for 10 min, 45°C for 10 min, and 73°C for 15 min. After *Hin*dIII digestion, the resulting probes were purified by electrophoresis in 8.3 M urea/4% polyacrylamide gels.

5′-Rapid Amplification of cDNA Ends (RACE). 5′-RACE was done by a modification of a published method (14). Human caudate poly(A)$^+$ RNA was reverse-transcribed with PS6 primer (Fig. 1*B*), and a poly(dA) tail was added with terminal deoxynucleotidyltransferase (Stratagene). The product was subjected to PCR using three primers: 3Ex1 as the 3′ primer (50 pmol), which is complementary to nucleotides −970 to −987; adaptor (50 pmol), 5′-GCCGCATGC-GAATTCACC-3′; and adaptor-$(dT)_{17}$ (6 pmol). PCR was set at 92°C for 5 min, 72°C for 5 min, 55°C for 10 min, 73°C for 30 min, followed by 20 cycles of 94°C for 1 min, 50°C for 1 min, and 73°C for 3 min; 20 cycles of 94°C for 1 min, 50°C for 2 min, and 73°C for 3 min; and finally 73°C for 15 min. PCR products were treated with *Escherichia coli* DNA polymerase I Klenow fragment and phage T4 polynucleotide kinase and then ligated with pUC19, giving a partial cDNA library. This library was screened with primer 5Ex1 (identical to nucleotides −1040 to −1023) as a probe, and filters were washed with 2× standard saline citrate at 42°C for 15 min. Among the nearly 700 clones screened, the 19 positive ones were sequenced.

Plasmid Constructions and Transient Expression Assays. Four overlapping fragments of the *Sac* I–*Pst* I fragment in pD1G3 (see Fig. 4) were subcloned in pCAT-Basic plasmid to make D_{1A} promoter–CAT gene chimeras. pCAT-Basic, which lacks a promoter and an enhancer, was used as a negative control, and pCAT-Control (Promega), which has both the promoter and the enhancer of simian virus 40, was used as a positive control for chloramphenicol acetyltransferase (CAT) assays.

The murine neuroblastoma cell lines NS20Y and NB41A3, the rat C6 glioma, and human hepatocellular carcinoma Hep G2 were all cultured in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 10% CO_2. NS20Y was a gift from Marshall Nirenberg (National Institutes of Health). The other cell lines were obtained from American Type Culture Collection. Transfections were carried out by the calcium phosphate coprecipitation method (15) with 10 μg of the test pCAT plasmid and 10 μg of the expression vector pRAS-βGAL (39) per 100-mm culture plate to correct for transfection efficiency among plates. All plasmids used in transfections were purified by two CsCl centrifugations. After transfection, cell lysate [normalized according to β-galactosidase activity (13)] was incubated with 0.15 μCi of [^{14}C]chloramphenicol (Amersham), 0.5 mM acetyl-CoA, and 0.25 M Tris·HCl (pH 7.8) in a final volume of 160 μl at 37°C for 1 hr. The incubation mixture was extracted with

ethyl acetate and analyzed by thin-layer chromatography. Radioactivity was quantitated by a liquid scintillation counter. Each transfection experiment was repeated at least twice and gave reproducible results.

RESULTS AND DISCUSSION

Nucleotide Sequence of the 5′ Flanking Region of the Human D_{1A} Gene. Screening a human genomic library with a rat D_{1A} cDNA yielded 11 positive plaques. The 5.3-kb *Sac* I fragment of the most positive clone was subcloned in pGEM-3Zf(−) to give plasmid pD1-5.3 (Fig. 1*A*). The restriction map of the portion of pD1-5.3 downstream from the *Eco*RI site and the nucleotide sequence between the *Eco*RI and *Pst* I sites are identical to the reported sequence of the human D_{1A} gene (8–10). The 2.3-kb *Sac* I–*Pst* I fragment was subcloned into pGEM-3Zf(−), giving plasmid pD1G3, and sequenced (2336 bp total) (Fig. 1*B*). This portion of the gene spans nucleotides −2571 to −236 relative to the adenosine in the first ATG of the open reading frame (8–10).

Exon/Intron Organization of the Human D_{1A} Gene. Initial attempts to define the transcription start site were made with S1 mapping using PS4 (−373 to −402; Fig. 1*B*) as the primer for synthesizing a 5′-end-labeled S1 probe. This experiment showed that the protected band extended to the adenosine at −486 (data not shown). However, evaluation of the sequence in the immediate vicinity of this nucleotide revealed high homology to the consensus sequence for a splice acceptor site (16). Furthermore, sequences homologous to a splice donor site were found at bases −549, −599, and −909. Thus, S1 mapping using a 5′-end-labeled probe could have detected the 5′ end of an exon. To verify the intron/exon structure of this gene, reverse transcription–PCR experiments were conducted using human caudate poly(A)$^+$ RNA (Fig. 2). Six primers (J1–J6) were designed around these intron donor and acceptor sites to be used as 5′ primers in conjunction with a 19-mer 3′ primer (D1-3′) located at bases −408 to −426 (Fig. 2*A*). As expected, a specific PCR product was obtained with J1, which is located downstream to the acceptor site at −484 (Fig. 2*B*). Similarly, primer J2, which is located upstream of the donor site at −599, gave a specific band of a size consistent with an internal intron of around 116 bp. In addition, J3, which includes sequences upstream of −599 and downstream to −484, gave a band suggesting that −599 is indeed used as a donor site. On the other hand, no PCR product could be amplified by using J4, which is made of sequences upstream of the putative donor site at −549 and downstream to −484, ruling out the use of −549 as a donor site. Similarly, J5, which spans the sequences upstream of the possible splice donor site at −909 and downstream to −484, gave no band, ruling out the use of −909 as a donor site. Finally, J6 gave no PCR band, because only its 5′ portion is complementary to the mRNA, while its 3′ portion is complementary to the intronic region, which does not exist in the mRNA. These findings suggest that intron I of this gene is 116 bp long, extending from −599 to −484. The presence of exon I sequence in mRNA was verified by the amplification of a specific PCR product from primers 5Ex1 (identical to −1040 to −1023, 5′-ACAGGCTCCTGAGAGGTC-3′) and 3Ex1 (complementary to −970 to −987, 5′-GGCCTTCAGCCCTA-CAGA-3′) (data not shown). Thus, the human D_{1A} gene, which was previously thought to be intronless (8–10), has a small intron in its 5′ noncoding region.

The presence of an intron in the 5′ noncoding region but not in the translated portion has also been reported for muscarinic acetylcholine receptor genes (17, 18). Several other G-protein-coupled receptors are known to contain introns, including the rat and human D_2 (19–22), rat D_3 (23), and human D_4 (24) dopamine receptor genes, the opsin gene (25), and the rat substance P receptor gene (26). Both the D_{1A} and D_2 genes, the two main dopamine receptors expressed in the striatum, have an intron in their 5′ noncoding region. The functional significance of such an intron remains to be investigated.

Determination of the Transcription Start Site of the Human D_{1A} Gene. Transcription initiation site was determined by S1 nuclease analysis and 5′-RACE. With S1 probes made with primer PS5 (−600 to −629) (Fig. 3) or PS6 (−937 to −966) (data not shown), both of which are located upstream of intron I, diffuse bands were obtained, suggesting that tran-

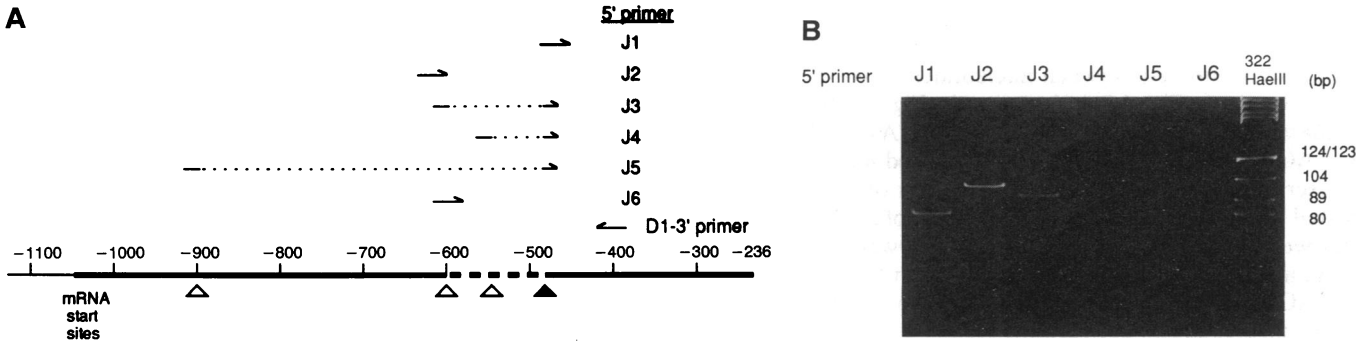

FIG. 2. Characterization of intron I by PCR. (*A*) Strategy employed to detect the intron by reverse transcription–PCR. The nucleotide sequence of the 5′ noncoding region of the D_{1A} gene is shown as a line with the same numbering as in Fig. 1*B*. The thick portion of the line represents the exons and the interrupted part represents intron I. Possible intron donor sites and acceptor site determined from the nucleotide sequence are shown by open and filled triangles, respectively. One 3′ primer and six 5′ primers were designed and are shown as horizontal arrows indicating their location and orientation. Solid lines are for sequences included in primers and interrupted lines are for gaps in the primer sequence. D1-3′ is complementary to bases −408 to −426 (5′-CCTTCTGAGACTCAGCTGA-3′), J1 extends between −485 and −468 (5′-AGGCGTTTGGAGAGCTGC-3′), J2 spans bases −620 to −603 (5′-GAGAGACGACCCCAAGGC-3′), and J6 covers −611 to −594 (5′-CCCCAAGGCAAGGTAGGT-3′). Three additional 5′ primers stretching over each of the three possible splice junctional sequences were designed: J3 spans nucleotides −612 to −600 and −483 to −479 (5′-ACCCCAAGGCAAGGCGTT-3′), J4 covers bases −562 to −550 and −483 to −479 (5′-GGTTGGGCTTGACGCGTT-3′), and J5 includes bases −922 to −910 and −483 to −479 (5′-AGAAGCTGCCAAGGCGTT-3′). (*B*) Result of reverse transcription followed by PCR of D_{1A} mRNA from human caudate. The PCR mixture contained 10 μl of template cDNA, 50 pmol of primer D1-3′, 50 pmol of one of the six 5′ primers, 0.5 mM each dNTP, 5 units of *Taq* DNA polymerase and 1 unit of Perfect Match DNA polymerase enhancer (Stratagene) in 50 μl of *Taq* polymerase buffer. PCR was set at 73°C for 10 min, followed by 20 cycles of 94°C for 1 min, 50°C for 1 min, and 73°C for 3 min, 20 cycles of 94°C for 1 min, 50°C for 2 min, and 73°C for 3 min, and finally 73°C for 15 min. PCR products were electrophoresed in 10% polyacrylamide gel. *Hae* III-digested pBR322 plasmid was used as size markers in the far right lane (bp, base pairs). Specificity of all PCR products was examined by restriction analysis, and the exon I/exon II junctional sequence in the cDNA was verified by sequencing both strands of the PCR product of J2 and D1-3′.

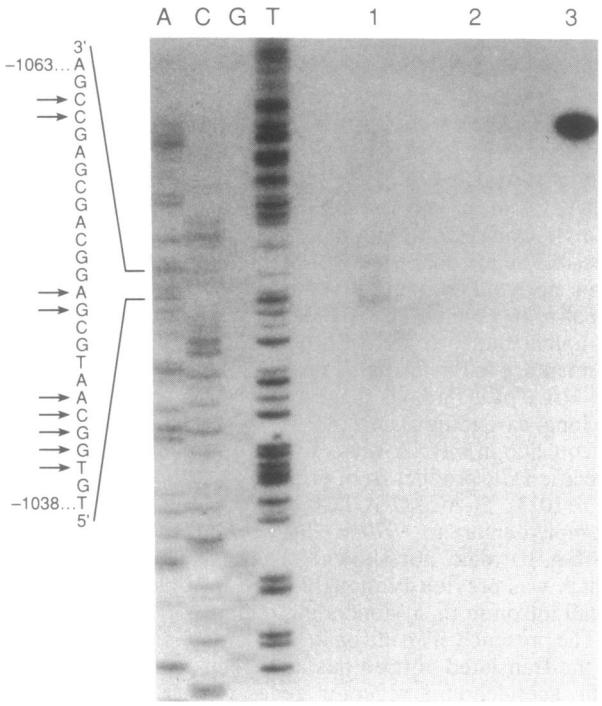

FIG. 3. S1 nuclease analysis. Single-stranded, 5'-end-labeled DNA probe made with PS5 primer (Fig. 1*B*) by using the genomic D_{1A} clone as template was boiled for 10 min with 50 μg of total RNA from human caudate (lane 1) or 50 μg of yeast tRNA (lane 2) in 25 μl of hybridization buffer (40 mM Pipes, pH 6.4/400 mM NaCl/1 mM EDTA, pH 8.0/80% formamide), cooled gradually, and annealed overnight at 30°C. Following hybridization, 300 μl of S1 nuclease buffer (0.28 M NaCl/50 mM NaOAc, pH 4.5/4.5 mM $ZnSO_4$ with calf thymus DNA at 20 μg/ml) and 300 units of S1 nuclease (United States Biochemical) were added and the mixture was incubated at 30°C for 60 min. The protected fragments were electrophoresed in 6% polyacrylamide gel containing 8.3 M urea. Lane 3, probe alone without digestion. The DNA sequence of the negative strand obtained with the same primer (PS5) used for preparing S1 probe is shown at left. Reproducible results were obtained with a probe made with the more upstream primer PS6. Arrows, transcription start sites suggested by S1 mapping performed with the PS6-primed probe.

scription is initiated between nucleotides −1061 and −1040 (arrowheads in Fig. 1*B* and arrows in Fig. 3). In addition, sequencing of the 5' ends of D_{1A} cDNAs made by the 5'-RACE method using primer PS6 showed no evidence for transcription starting at a point upstream of the sites suggested by S1 mapping. In fact, the 5' ends of all the 19 clones that were products of the RACE reaction were at the adenosine at −1040. Thus, the findings of both S1 mapping and 5'-RACE indicate that there is no exon upstream of −1061. Minor discrepancies between the results of the two methods are most likely due to intrinsic differences between the two procedures. A third method, primer extension, was attempted repeatedly without success, probably due to the relatively low abundance of the D_{1A} message in the striatum. This low abundance may also explain the faint bands obtained with S1 mapping, particularly using primer PS6. We conclude that the 5' untranslated region of the human D_{1A} transcript is about 920 bases long, similar to the length of the 5' untranslated region of the insulin-like growth factor receptor gene (27), and exon I of the D_{1A} gene is about 440 bases. The present assignment of transcription initiation sites, the presence of a small intron, and the known 3' extent of the D_{1A} cDNA are all consistent with the previously reported mRNA length, ≈4 kb (8–10).

Sequence analysis of the region upstream of the transcription initiation site revealed that the promoter region of this

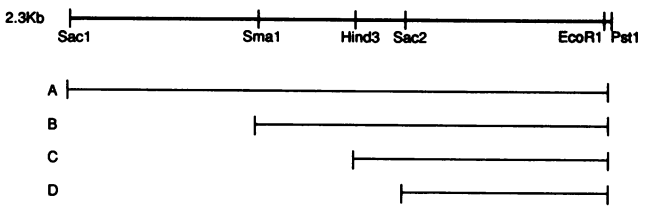

FIG. 4. Strategy for subcloning fragments of the D_{1A} gene 5' flanking region. Four fragments (A–D) of the D_{1A} gene 5' flanking region were subcloned in the 5' to 3' orientation between the *Hin*dIII and *Pst* I sites of pCAT-Basic plasmid (Promega) upstream of the translation initiation site of the CAT gene. The 5' ends of fragments A, B, and D were blunt-ended with Klenow polymerase and ligated with the filled-in *Hin*dIII site of the vector. Insert length and orientation were verified by restriction analysis and sequencing. The resulting constructs were designated as pCAT-HD1G-A through -D.

gene has neither a TATA box nor a CAAT box. The sequence ATTGG, which is the reverse orientation of a CAAT box, is found at nucleotide −1322; however, this is not the expected position of a CAAT box relative to the transcription initiation site. This gene is highly rich in G+C content, reaching 80% in some portions. Analysis of the sequence revealed no open reading frame of significant length upstream of the initiator methionine codon at position +1. Homology analysis with known enhancer consensus sequences revealed that the D_{1A} gene 5' flanking region contains putative sequences for several transcription factor binding sites. It has several putative Sp1 binding sites (Fig. 1*B*) as well as inverted G·C boxes (GGCGGG) (28, 29) at positions −2148, −1635, −1121, and −1089. Sequences homologous to an AP1 binding site (30) are found at −2083 and −543, the latter being in the intron. In addition, a putative cAMP response element (ACGTCA) is found in the intron at −548 (minus strand) and there are multiple possible AP2 sites throughout the sequenced fragment (Fig. 1*B*) (31). The functional significance of these putative transcription factor binding sites in the expression of the D_{1A} gene remains to be determined.

Transcription of the human D_{1A} gene from a TATA-less promoter and other sequence features of this region, including multiple transcription start sites, high G+C content, and multiple Sp1 binding sites, are reminiscent of "housekeeping" genes (32). However, the D_{1A} gene is expressed in a tissue-specific manner and is regulated (see below). A large number of tissue-specific genes, including some expressed only in neural cells, have similar housekeeping-type promoters: the nerve growth factor receptor gene (33), the gene

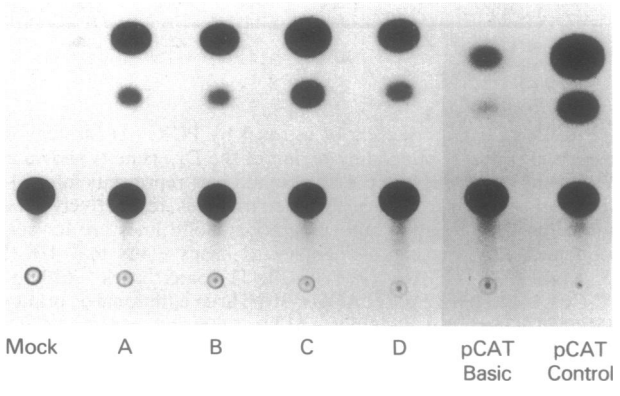

FIG. 5. CAT assay with the murine neuroblastoma cell line NS20Y. Names of plasmids used for transfection are shown. A through D are the fragments shown in Fig. 4, cloned into pCAT-Basic vector. Lysate from cells subjected to calcium phosphate coprecipitation without any DNA was used for "Mock" experiment.

Table 1. Stimulation of CAT activity in four cell lines

Clone	Relative CAT activity			
	NS20Y	NB41A3	C6	Hep G2
pCAT-Control	26.9	50.9	22.5	22.8
pCAT-HD1G-A	7.4	0.71	1.1	0.22
pCAT-HD1G-B	6.0	0.71	1.2	0.036
pCAT-HD1G-C	18.5	2.0	3.5	0.12
pCAT-HD1G-D	5.7	1.1	2.2	0.054

CAT assays were carried out in four cell lines of different origins. Deletion mutants are indicated on the left. Transfection efficiencies were normalized according to β-galactosidase activity and the results are expressed as fold acetylation compared with the negative control plasmid pCAT-Basic in each cell line. All transfections were repeated at least twice, yielding similar results. Origins of cell lines are as follows: NS20Y and NB41A3, mouse neuroblastoma; C6, rat glioma; Hep G2, human hepatoblastoma.

encoding the α subunit of the regulatory protein G_o (34), the synapsin I gene (35), and the protooncogene *Pim*-1 (36).

Cell Type-Specific Promoter/Enhancer Activity of the 5′ Flanking Region of the Human D_{1A} Gene. To localize the regulatory regions in the 5′ upstream portion of the human D_{1A} gene, four restriction fragments, A to D (Fig. 4), were subcloned into pCAT-Basic plasmid, yielding clones pCAT-HD1G-A, through -D, respectively. In NS20Y cells, which are known to express D_{1A} dopamine receptors (37, 38), all subclones had some transcriptional activity, with fragment C being the strongest (Fig. 5 and Table 1). Thus, the *Hin*dIII–*Sac* II fragment (−1340 to −1102) has a powerful positive modulator, whereas the *Sma* I–*Hin*dIII fragment (−1730 to −1341) appears to possess a negative modulatory action. S1 nuclease mapping using RNA from NS20Y cells transfected with pCAT-HD1G-A and -C indicated that transcription of the CAT gene in NS20Y cells is indeed driven by the D_{1A} gene promoter and that transcription is initiated at the same points as *in vivo* in the striatum (data not shown). To assess the cell type specificity of these regulatory elements, three other cell lines—NB41A3, C6, and Hep G2, all derived from different tissues—were transfected with each of the CAT constructs (Table 1). All deletion mutants were markedly less effective in modulating the transcription of the heterologous CAT gene in these cell lines than in NS20Y cells. Fragment C, which was nearly as effective in inducing gene transcription as the positive control plasmid in NS20Y cells, had much less activity in the other cell lines. Even the smallest fragment, D, demonstrated cell type-specific promoter activity. In the neuroblastoma cell line NB41A3, which expresses the D_2 but not the D_{1A} gene (37), the transfected D_{1A} promoter–CAT gene was essentially silent. Thus, the transcriptional capability of the 5′ upstream region of the human D_{1A} gene is largely limited to cells that have the nuclear machinery to interact with its promoter/enhancer elements and to modulate its transcription.

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